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A Role for the Roof Plate and Its Resident TGFβ-Related Proteins in Neuronal Patterning in the Dorsal Spinal Cord

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Summary

Distinct neuronal cell types are generated at characteristic times and positions in the dorsal horn of the spinal cord. We provide evidence that the identity and pattern of generation of dorsal neurons depend initially on BMP-mediated signals that derive from the epidermal ectoderm and induce dorsal midline cells of the roof plate. Roof plate cells provide a secondary source of TGFβ-related signals that are required for the generation of distinct classes of dorsal interneurons. These inductive interactions involve both qualitative and quantitative differences in signaling by TGFβ-related factors and temporal changes in the response of neural progenitor cells.

Introduction

Neurons in the dorsal horn of the spinal cord contribute to the central processing of somatic sensory stimuli. In the adult spinal cord, several classes of dorsal horn neurons have been defined on the basis of their peripheral receptive properties, laminar position, and axonal projections (Brown, 1981). These neurons derive from progenitor cells located in the dorsal half of the neural tube (Nornes and Das, 1974; Altman and Bayer, 1984; Eide and Glover, 1997), but the source and identity of signals involved in their differentiation has not been defined.

The dorsal neural tube also contains the progenitors of neural crest cells that subsequently migrate into the periphery (Bronner-Fraser, 1994). Neural crest differentiation appears to be initiated by a short-range signal from the epidermal ectoderm (Moury and Jacobson, 1990; Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995). The induction of neural crest cells is mimicked by members of the TGFβ family, notably BMP4 and BMP7 (Basler et al., 1993; Liem et al., 1995), both of which are expressed transiently in the epidermal ectoderm (Liem et al., 1995; Schultheiss et al., 1997). BMPs represent the only known class of secreted proteins with the ability to generate neural crest cells, but it has yet to be demonstrated that they are required in this inductive process.

Although implicated in the induction of neural crest cells, the epidermal ectoderm may not be a direct source of signals that induce dorsal horn neurons. Many of the neurons that populate the dorsal horn are generated only at late stages (Nornes and Das, 1974; Oppenheim et al., 1988), after the onset of neural crest cell generation (Newgreen and Erickson, 1986). At these late stages, the epidermal ectoderm is no longer in contact with the dorsal neural tube and the ectodermal expression of *BMP4* and *BMP7* has been down-regulated (Liem et al., 1995; Watanabe and Le Douarin, 1996). One potential source of signals that might control the generation of dorsal horn neurons is the roof plate, a specialized dorsal midline cell type (Altman and Bayer, 1984). Roof plate cells express several classes of secreted signaling factors including BMPs (Basler et al., 1993; Liem et al., 1995; Watanabe and Le Douarin, 1996), Wnts (Hollyday et al., 1995), and Ephrins (Gale et al., 1996). However, the functions of the roof plate and its resident signaling factors in neural patterning have not been examined.

In this study, we have investigated the role of the roof plate in the induction and patterning of interneurons in the dorsal spinal cord. We have defined two classes of interneurons, termed D1 and D2, that are generated close to the roof plate. The generation of D1 and D2 neurons can be induced by signals from the roof plate that are mediated by members of the TGFβ family. The distinct identities and positions of generation of these two classes of neurons appear to involve both qualitative and quantitative differences in signaling by TGFβ-related molecules and temporal changes in the response of dorsal progenitor cells. The differentiation of roof plate cells themselves depends on an earlier BMP-mediated signal from the epidermal ectoderm. These results provide evidence that the patterning of neurons in the dorsal horn of the spinal cord requires a cascade of TGFβ inductive signaling that is initiated by the epidermal ectoderm and propagated by the roof plate.

Results

LIM Homeodomain Proteins Define Distinct Classes of Dorsal Interneurons

To define markers of dorsal interneurons, we analyzed the pattern of expression of LIM homeodomain proteins, a class of transcription factors that distinguish other spinal neurons (Tsuchida et al., 1994). One subset of neurons, generated from stage 19, close to the roof plate, expresses the LH2 proteins (Figure 1A and data not shown). We define these cells as D1 neurons (Figure 1E). Between stages 24 and 27, D1 neurons are found more ventrally (Figures 1B and 1C), and by stage 29, they are found in deep laminae in the dorsal spinal cord (data not shown). Many neurons that are generated at a dorsal position migrate ventrally and settle in the deep dorsal horn (Carpenter and Hollyday, 1992; Leber and Sanes, 1995), and it is likely that D1 neurons are included in this population.

A distinct class of dorsal interneurons, termed D2 neurons, expresses Isl1 (Figures 1B and 1C; data not

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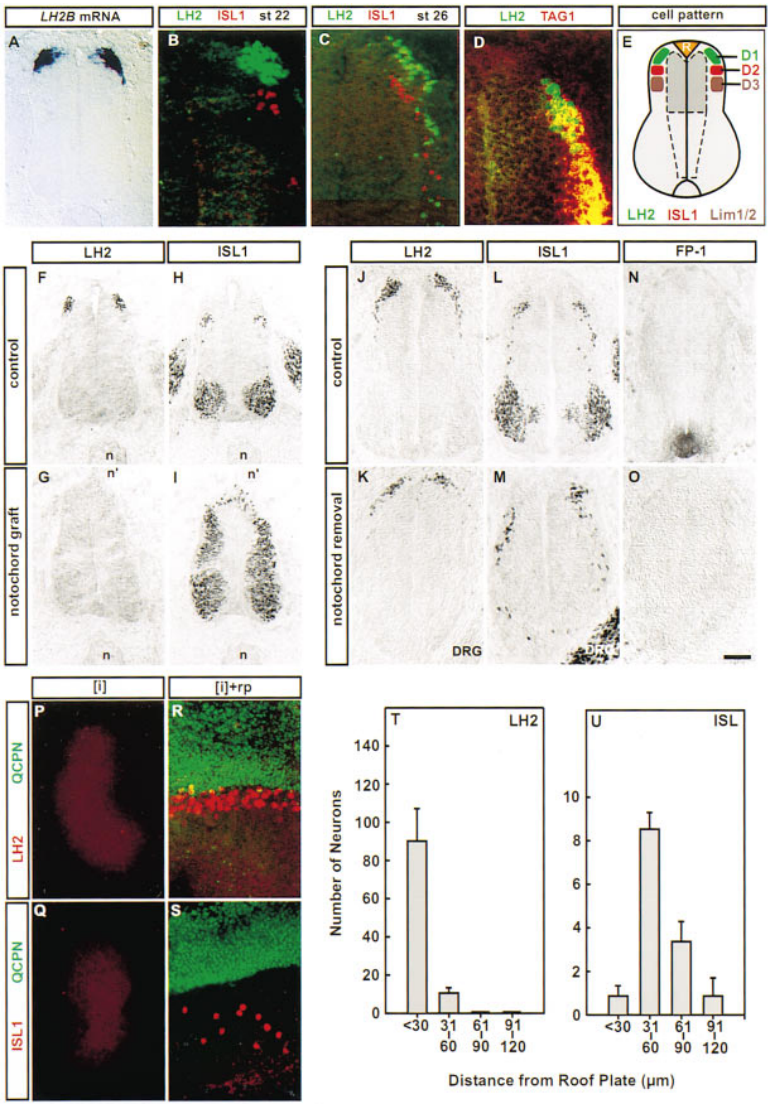

Figure 1. Identification and Regulation of Dorsal Interneuron Differentiation

(A) Section of stage 24 spinal cord showing localization of *LH2B*. (B and C) LH2 expression (green) and Isl1 expression (red) at stages 22 (B) and 26 (C). (D) Section through stage 25 spinal cord showing that most LH2 neurons (green) coexpress the surface glycoprotein TAG-1/Axonin-1 (red). LH2 is expressed before TAG-1/Axonin-1. Ventral TAG-1 cells do not express LH2. (E) Position of generation of D1, D2, and D3 interneurons. The position of the roof plate (R) is shown. (F) LH2 neurons in dorsal spinal cord in a stage 24 embryo rostral to the region at which a notochord has been grafted. (G) Section through embryo in (F) at a level at which a dorsal notochord graft (n′) is present. No LH2 (D1) neurons are detected.

(H) Section adjacent to that in (F) showing expression of Isl1 in motor neurons and in dorsal (D2) neurons. (I) Section adjacent to that in (G), showing dorsal Isl1/2 neurons after a dorsal notochord graft. (J) Expression of LH2 in dorsal spinal cord in a stage 25 embryo, rostral to the level of notochord removal. (K) Section from the embryo in (J) at a level lacking a notochord showing the persistence of LH2 neurons. The position of generation and number (~40 cells/section) of LH2 neurons is similar to that at levels at which the notochord is present. (L) Expression of Isl1 in a section serial to that in (J). A notochord is present at this level. (M) Section serial to that in (K) showing the depletion of ventral Isl1 neurons at levels lacking a notochord. Isl1 neurons are generated in the dorsal spinal cord and their number (~25 cells/section) and position are similar to those at levels at which the notochord is present (L). (N and O) Expression of the floor plate marker FP-1 at a level rostral to that at which the notochord had been removed (N) and lack of FP-1 expression at the level of notochord removal (O). DRG = dorsal root ganglion. Scale bar = 50 μm. (P–S) Confocal images of stage 10 [i] explants cultured for 48 hr with stage 20–27 quail roof plate ($QCPN^+$; green). In (P) and (Q), [i] explants do not give rise to LH2 neurons (P) (n = 7) or Isl1 neurons (Q) (n = 7). In (R), roof plate (green) induces LH2 neurons (137 ± 20 cells/explant; n = 7). In (S), roof plate induces Isl1 neurons (11 ± 4 cells/explant; n = 7). $Isl1^+$ cells do not express Isl2 (data not shown). (T and U) Position of generation of LH2 (T) and Isl1 (U) neurons as a function of distance from the roof plate. Mean ± SE; n = 6.

shown; Tsuchida et al., 1994). Neither D1 nor D2 neurons express Lim1/2, a marker of D3 neurons (Figure 1E; data not shown). D2 neurons are generated over the same time period as D1 neurons but initially occupy a more medial and ventral position (Figures 1B and 1C) and settle in a more ventral position (data not shown). D1 but not D2 neurons coexpress TAG1/Axonin1 (Figure 1D and data not shown), a marker of dorsal commissural neurons (Dodd et al., 1988; Zuellig et al., 1992).

To explore the factors that control the identity and position of differentiation of dorsal interneurons, we examined first whether their generation is influenced by the notochord, a source of signals that ventralizes the neural tube (Tanabe and Jessell, 1996). Chick notochord grafts placed adjacent to the dorsal neural tube suppressed the generation of D1 neurons (Figures 1F and 1G) under conditions in which $Isl1/2^+$ motor neurons were generated at ectopic dorsal positions (Figures 1H and 1I; data not shown). These results argue against the idea (Artinger and Bronner-Fraser, 1992) that the generation of dorsal commissural neurons is resistant to notochord signals.

We next examined whether a long-range action of notochord-derived signals normally restricts the position of generation of D1 and D2 neurons. To test this, we removed a segment of the notochord that underlies the caudal neural plate at stage 10 and permitted embryos to develop for 72 hr. Neither the number of D1 and D2 neurons nor their position was markedly affected by notochord removal (Figures 1J–1M). The effectiveness of notochord removal was established by the absence of expression of the floor plate marker, FP-1 (Figures 1N and 1O) and by the loss of $Isl1/2^+$ motor neurons (Figures 1L and 1M). Thus, the action of notochord-

derived signals does not appear to contribute to the dorsal position at which D1 and D2 neurons are generated, raising the possibility that cells at or near the dorsal midline of the spinal cord induce and control the position of generation of dorsal interneurons.

The Roof Plate and Its Resident TGFβ-Related Proteins Induce D1 and D2 Neurons

To test whether signals from the roof plate induce the generation of D1 and D2 neurons, stage 10 intermediate neural plate ([i]) explants were cultured for 48 hr, alone or in the presence of stage 20–26 quail roof plate. Neither D1 nor D2 neurons were generated when [i] explants were cultured alone (Figures 1P and 1Q). In contrast, culture of [i] explants with roof plate resulted in the generation of D1 neurons close to the junction with roof plate (Figures 1R and 1T) and of D2 neurons at a more distal position (Figures 1S and 1U). Intermediate regions of stage 20–26 quail spinal cord did not induce D1 or D2 neurons (data not shown). These results show that the roof plate is a source of signals that can induce both D1 and D2 neurons. They show also that the positions at which D1 and D2 neurons are normally generated relative to the roof plate can be recapitulated in vitro.

We next addressed the identity of roof plate–derived signals that might mediate the induction of D1 and D2 neurons. Our studies have focused on the TGFβ family since two members of this family, *BMP4* and *DSL1*, are expressed by roof plate cells (Basler et al., 1993; Liem et al., 1995). We first determined the profile of *BMP* gene expression by the roof plate at stage 18, just prior to the onset of generation of D1 and D2 neurons. *BMP4* and *BMP5* were expressed at high levels by roof plate cells (Figures 2A and 2B). The expression of both *DSL1* and *BMP7* was also detected, but initially over a wider domain of the dorsal spinal cord (Figures 2C, and 2D; Basler et al., 1993; Liem et al., 1995). Neither *BMP2* nor *BMP6* are expressed in the spinal cord at this stage (data not shown). However, *Activin B* is expressed by the roof plate and adjacent cells (Figure 2E). Thus, cells in and adjacent to the roof plate express, in nested domains (Figure 2F), five members of the *TGFβ* superfamily over the period that D1 and D2 neurons are generated.

To examine whether TGFβ-related proteins mimic the activity of the roof plate, BMP and Activin proteins were tested for their ability to induce D1 and D2 neurons in stage 10 [i] explants grown for 48–72 hr. Addition of BMP4, BMP5, BMP7, or DSL1 induced many D1 neurons (Figures 2G–2J and 2S). In contrast, none of these BMPs induced D2 neurons (Figures 2M–2P and 2S; data not shown). Experiments described below, however, show that BMPs can, under different conditions, induce D2 neurons.

Activins exhibit activities distinct from those of BMPs in several embryonic tissues (Smith, 1993). The detection of *Activin B* in and around the roof plate (Figure 2E) led us to examine whether Activins could induce D1 or D2 neurons. Since the Activin A and B proteins exhibit equivalent biological activities and potencies (Mason et al., 1989) and cells in neural plate explants respond to Activin A (Pituello et al., 1995), we cultured stage 10 [i] explants in the presence of recombinant Activin A. Many D2 neurons were induced after 48 hr (Figures 2Q and 2T). Activin A also induced D1 neurons (Figures 2K and 2T), although the level of LH2 expression per cell was lower than that obtained in response to BMPs (Figure 2K). In addition, the number of D1 neurons decreased at high Activin A concentrations, whereas the number of D2 neurons increased (Figure 2T). These results raise the possibility that qualitative distinctions in the signaling activities of the two subclasses of TGFβ-related proteins detected in and around the roof plate contribute to the specification of D1 and D2 neuron identities.

The onset of expression of *BMPs* precedes that of *Activin B* in the dorsal neural tube (Liem et al., 1995 and data not shown), and later, their domains of expression overlap (Figure 2F). These findings raise the issue of the fate of dorsal progenitor cells exposed to both BMPs and Activin A. To address this, we exposed stage [i] explants for 48 hr simultaneously to submaximal concentrations of BMP4 and Activin A. The number of D1 neurons induced was close to the sum detected in explants exposed separately to each inducer (Figure 2U). In contrast, the generation of D2 neurons was decreased by ~80% in comparison to explants exposed solely to Activin A (Figure 2V). Exposure of [i] explants to BMP4 alone for 24 hr, prior to the addition of Activin A, inhibited the generation of D2 neurons by 95% (data not shown). Thus, joint or prior exposure of cells to BMPs markedly reduces the incidence of D2 neuron generation in response to Activin A.

These results pose the problem of how progenitor cell differentiation into D2 neurons is achieved. D2 neurons are generated ventral to D1 neurons in vivo and distant to D1 neurons in [i] explant–roof plate conjugates. Since early exposure of neural cells to BMPs suppresses the potential for D2 neuron generation, it is possible that D2 neurons derive from dorsally located progenitors that escape early BMP signaling but are subsequently exposed to TGFβ-related signals. To address this, stage 10 [i] explants were matured for 24 hr in the absence of BMPs. Cells in such explants were still able to respond to Activin A with the generation of D2 neurons (Figure 6O). We found in addition that cells in such explants responded to BMP exposure with the generation of D2 as well as D1 neurons (Figures 3B and 3C). Indeed, exposure to low concentrations of BMP4 induced D2 neurons in the absence of D1 neurons (Figures 3B and 3J), whereas higher BMP4 concentrations induced many D1 neurons but still some D2 neurons (Figures 3C and 3J).

These findings led us to examine whether dorsal progenitor cells that have been matured in vivo without exposure to *BMP* signaling behave similarly. To obtain such progenitors, we isolated explants from the ventralmost region of the dorsal neural tube of stage 15 embryos, ventral to the domain of *BMP* gene expression (see Figure 2F and Liem et al., 1995). Cells in such explants gave rise neither to D1 nor D2 neurons when grown alone (Figures 3D and 3G), but generated D2 and D1 neurons upon exposure to roof plate signals (Figures 3E and 3H), to BMP4 (Figures 3F and 3I) and to Activin A (data not shown).

These results suggest that the early history of exposure of dorsal progenitor cells to BMP signals is a factor

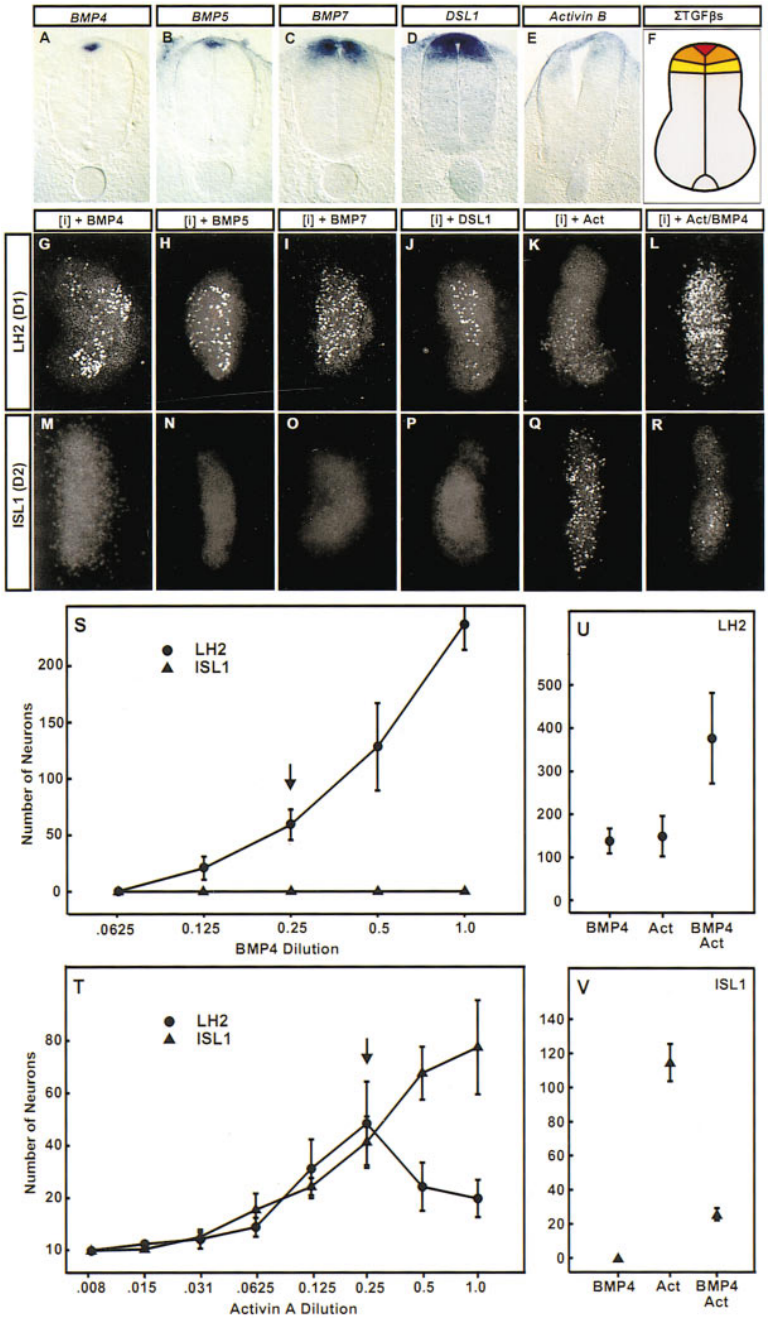

Figure 2. Induction of D1 and D2 Neurons by BMPs and Activin

(A–E) Expression of *BMPs* and *Activin B* mRNA in stage 18 chick spinal cord. (F) Nested expression of *BMPs* and *Activin B*. Red: *BMP4*, *BMP5*, *BMP7*, *DSL1*, and *Activin B*; dark orange: *BMP7*, *Dsl1*, and *Activin B*; light orange: *DSL1* and *Activin B*. (G–J) Stage 10 [i] explants cultured for 48 hr with BMPs generate LH2 (D1) neurons (BMP5, 36 ± 2 cells; BMP7, 96 ± 25 cells, DSL1 [10 pM], 62 ± 12 cells, n = 7). (K) Stage 10 [i] explants cultured for 48 hr with Activin A (at a concentration shown by arrow in [T]) generate weakly labeled LH2 neurons. (L) Stage 10 [i] explant exposed to both BMP4 and Activin A generates LH2 neurons; see (U) for quantitation. (M–P) Stage 10 [i] explants exposed to BMPs do not generate Isl1 neurons (n = 7). (Q) Stage 10 [i] explants exposed to Activin A generate Isl1 neurons; see (T) for quantitation. (R) Stage 10 [i] explants exposed for 48 hr to both BMP4 and Activin generate few D2 neurons; see (V) for quantitation. (S) BMP4 induces LH2 neurons in stage 10 [i] explants but does not induce Isl1 neurons. Similar results are obtained with BMP5, BMP7, and DSL1 (data not shown). (T) Stage 10 [i] explants exposed to Activin A generate both LH2 and Isl1 neurons. $Isl1^+$ neurons induced by Activin A do not coexpress Isl2 (data not shown). (U–V) Exposure of [i] explants to a submaximal concentration of BMP4 and Activin A for 48 hr results in the generation of a near-additive number of LH2 neurons (U) but in a >80% decrease in Isl1 neurons, compared to Activin A alone (V). Mean ± SE; n = 4–8 explants.

in determining their subsequent neuronal fate. D2 neurons may derive from progenitor cells that escape early BMP signals but are exposed subsequently to Activin and/or to low concentrations of BMPs.

Requirement for TGFβ-Related Proteins in the Induction of Dorsal Interneurons by the Roof Plate

To address the requirement for TGFβ signaling by the roof plate in the generation of dorsal interneurons, we examined the effect of secreted inhibitors of BMP and Activin signaling on the inductive activity of the roof plate. Noggin exerts its activity by binding to and antagonizing the actions of BMP4 (Zimmerman et al., 1996). Follistatin binds to and inactivates Activin (Nakamura et al., 1990) and BMP7 (Yamashita et al., 1995).

We tested first the ability of Noggin and Follistatin to inhibit the induction of D1 neurons in response to BMP4, BMP7, DSL1, and Activin. The BMP4-mediated induction of D1 neurons in stage 10 [i] explants was blocked by Noggin but not by Follistatin (Figure 4A). Conversely, the BMP7-mediated induction of D1 neurons was blocked by Follistatin but not by Noggin (Figure 4A). The induction of D1 neurons by Activin was also blocked by Follistatin (data not shown). In contrast, the DSL1-mediated induction of D1 neurons was not blocked by Noggin or Follistatin, alone or in combination (Figure 4A and data not shown). Thus, the inhibitory

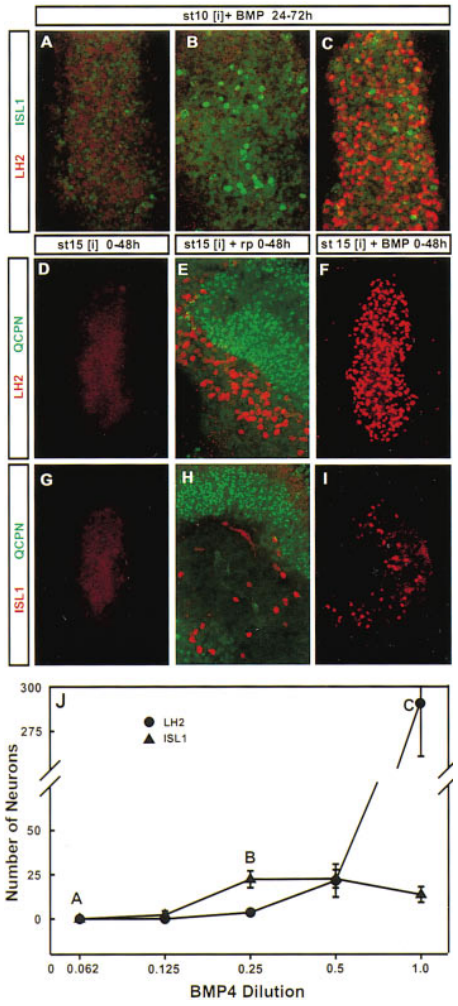

Figure 3. Neural Cells That Escape Early BMP Signals Respond Later to BMPs with the Generation of Both D1 and D2 Neurons

(A–C and J) Stage 10 [i] explants matured in the absence of BMP4 for 24 hr and subsequently cultured for an additional 48 hr with BMP4 give rise to Isl1 (green) and LH2 (red) neurons.

(D and G) Explants isolated from the ventral-most region of the dorsal neural tube of stage 15 embryos do not give rise to LH2 (D) or Isl1 (G) neurons.

(E and H) Quail roof plate (QCPN; green) induces LH2 (red in [E]) and Isl1 neurons (red in [H]) in stage 15 explants (LH2: 134 ± 28 cells/explant; Isl1: 19 ± 8 cells/explant; n = 3 explants).

(F) Stage 15 explants cultured for 48 hr with BMP4 generate LH2 neurons (56 ± 25 cells/explant; n = 4). Similar results were obtained with BMP7 (not shown).

(I) Stage 15 explants cultured with BMP4 generate Isl1 neurons (10 ± 1 cells/explant, n = 3). Similar results were obtained with BMP7 (not shown).

actions of Follistatin and Noggin appear selective for specific members of the BMP family.

We next examined whether the induction of D1 and D2 neurons by the roof plate is blocked by Noggin and Follistatin. Conjugates of stage 10 [i] explants and stage 26 quail roof plate were grown alone or with Noggin and Follistatin. The induction of D1 neurons in both stage 10 and 15 [i] explants by the roof plate was inhibited by 75%–80% in the presence of Noggin and Follistatin (Figures 4B and 4D–4F). The incomplete inhibition of D1 neuron generation may result from the actions of DSL1 or other BMPs that are insensitive to Noggin and Follistatin. In support of this, the induction of D1 neurons by epidermal ectoderm, which expresses BMP4 and BMP7 but not DSL1, was inhibited by 90% in the presence of Noggin and Follistatin (Figures 4B and 4G–4I). The induction of D2 neurons by the roof plate was inhibited by 40%–50% in the presence of Noggin and Follistatin (Figure 4C). The partial inhibition of D2 neurons may be a function of the low concentration threshold for induction of D2 neurons by BMPs (see Figure 3). If this is the case, the persistence of a low level of BMP signaling in the presence of Noggin and Follistatin might still be effective in generating a significant number of D2 neurons.

These findings provide evidence that the inductive activities of the roof plate are mediated in large part by the actions of TGFβ-related proteins that are sensitive to the blocking actions of Noggin and Follistatin.

Induction of Roof Plate Cells by BMP-Mediated Signals from Epidermal Ectoderm

The evidence that TGFβ-related signals from dorsal midline cells are both sufficient and required for the generation of distinct classes of dorsal interneurons prompted us to examine how the differentiation of roof plate cells themselves is controlled. Cells at the dorsal midline of the neural tube that give rise to the roof plate express the bZIP transcription factor MAFB (Cordes and Barsh, 1994; Kataoka et al., 1994; Eichmann et al., 1997) and PAX7 (Ericson et al., 1996) (Figure 5A), and expression of MAFB persists in the roof plate until at least stage 26 (data not shown).

To test whether dorsal midline cells are induced by signals from the epidermal ectoderm, stage 10 [i] explants were grown for 24 hr, alone or with epidermal ectoderm. Explants grown alone did not give rise to MAFB/PAX7 cells (Figure 5B), whereas explants cultured with epidermal ectoderm contained MAFB/PAX7 cells in the region of the explant close to the ectoderm (Figure 5C). The generation of MAFB/PAX7 cells was also induced by BMP4 and BMP7 (Figures 5D and 5E). The BMP4 concentration threshold for induction of dorsal midline cells was identical to that for neural crest cells (data not shown).

Since *BMP4* is selectively expressed by dorsal midline cells (Figure 5F) and appears to be involved in roof plate signaling, we used a RT–PCR assay to determine whether signals from the epidermal ectoderm are also sufficient to induce *BMP4*. In these experiments, rat epidermal ectoderm and chick *BMP4*-specific primers were used since the chick ectoderm itself expresses *BMP4* (Liem et al., 1995). In stage 10 [i] explants cultured alone for 24 hr, a low level of *BMP4* was detected (Figure 5G, lane 2) but explants grown in contact with rat epidermal ectoderm were induced to express a high level of *BMP4* (Figure 5G, lane 3). BMP4 and BMP7 mimicked the ability of the epidermal ectoderm to induce *BMP4* (Figure 5G, lanes 4 and 5). Chick neural plate isolated at stage 10 and assayed without culturing expressed

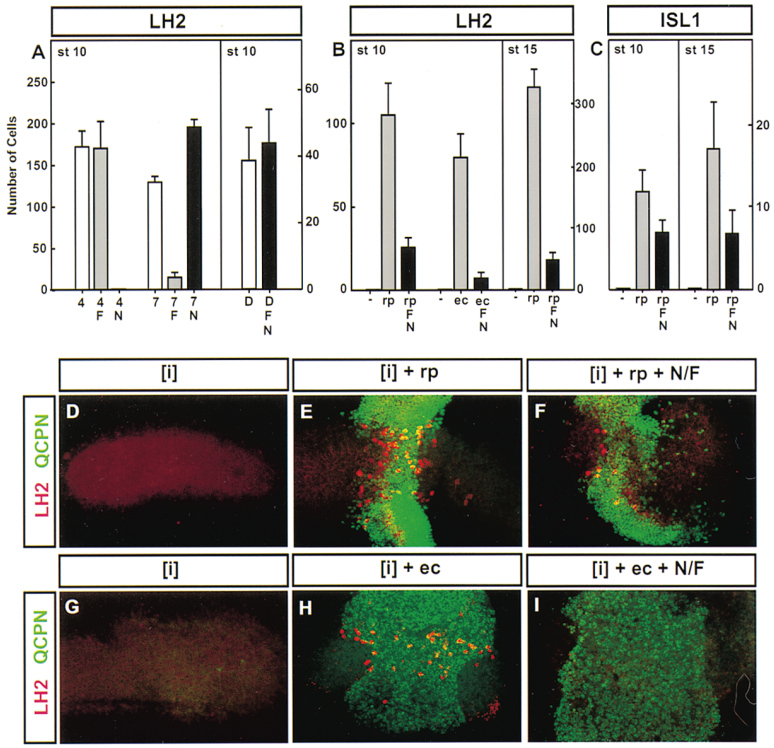

Figure 4. Requirement for TGFβ Signaling from the Roof Plate in the Induction of Dorsal Interneurons

(A) Stage 10 [i] explants cultured with BMP4 for 48 hr give rise to LH2 neurons and addition of Noggin (20 nM) but not Follistatin (85 nM) blocks the generation of LH2 neurons. Stage 10 [i] explants cultured with BMP7 for 48 hr generate LH2 neurons and addition of Follistatin (170 nM) but not Noggin (20 nM) blocks the generation of LH2 neurons. Noggin (20 nM) and Follistatin (85 nM) fail to block the DSL1 induction of LH2 neurons. Mean ± SE; n = 4.

(B) Stage 10 or stage 15 [i] explants grown with roof plate or epidermal ectoderm generate LH2 neurons. Addition of Noggin (20 nM) and Follistatin (85 nM) blocks the roof plate induction of LH2 neurons by 75%–80% and the epidermal ectoderm induction of LH2 neurons by ~90%. Mean ± SE; n = 10–17.

(C) Stage 10 or stage 15 [i] explants grown with roof plate generate Isl1 neurons. Addition of Noggin (20 nM) and Follistatin (85 nM) blocks the induction of Isl1 neurons by 40%–50%. Mean ± SE; n = 5–16.

(D–F) Noggin and Follistatin block induction of LH2 neurons by quail roof plate (green) (rp).

(G–I) Noggin and Follistatin block induction of LH2 neurons by quail epidermal ectoderm (green). Noggin and Follistatin do not block the generation of D3 interneurons or the Shh-N induction of motor neurons (data not shown).

(F) = Follistatin, (N) = Noggin, (ec) = ectoderm, (rp) = roof plate, (4) = BMP4, (7) = BMP7, and (D) = Dorsalin.

BMP4 in dorsal but not in intermediate or ventral regions (Figure 5G, lanes 6–8), indicating that the differentiation of dorsal midline cells is underway prior to neural tube closure. These results provide evidence that a BMP-mediated signal from the epidermal ectoderm can induce roof plate cells.

A Change in Competence of Neural Cells Controls the Timing of Neural Crest and Dorsal Interneuron Generation in Response to TGFβ-Related Signals

The differentiation of neural crest cells can be induced by BMPs present in the epidermal ectoderm (Liem et al., 1995). However, the onset of neural crest cell differentiation occurs shortly after neural tube closure, whereas D1 and D2 neuron generation occurs later and persists after neural crest cell generation has ceased (Newgreen and Erickson, 1986). These observations pose the question of why early dorsal progenitors respond to ectodermally derived BMPs with the generation of neural crest and not dorsal interneurons.

One possibility is that the level of BMPs provided by the epidermal ectoderm is below the threshold for induction of dorsal interneurons. To test this, we compared the concentration of BMP4 required for induction of HNK1$^+$ neural crest cells and D1 neurons in stage 10 [i] explants. BMP4 induced D1 neurons at a threshold concentration identical to that required to induce neural crest cells (data not shown). Moreover, [i] explants can generate D1 neurons when conjugated with epidermal ectoderm (Figure 4H). Thus, the failure to generate D1 neurons at stages soon after neural tube closure appears not to result from a low BMP concentration in the epidermal ectoderm.

We therefore examined whether the distinct phases of neural crest and dorsal interneuron generation might be controlled by a temporal change in the response of neural progenitor cells to BMPs. To test this, we exposed stage 10 [i] explants to a fixed concentration of BMP4 for a 24 hr period, either from 0 hr to 24 hr or from 24 hr to 48 hr, and analyzed the differentiation of neural crest cells and D1 neurons. Exposure of [i] explants to BMP4 from 0 hr to 24 hr induced premigratory (Slug$^+$) and migratory (HNK1$^+$) neural crest cells but not D1 neurons (Figures 6A–6D). In contrast, [i] explants grown alone for the first 24 hr and then exposed to BMP4, from 24 hr to 48 hr, generated D1 and D2 neurons but not neural crest cells (Figures 6E–6H and data not shown). Similarly, exposure of [i] explants to Activin A from 0 hr to 24 hr generated neural crest cells but no D1 neurons and very few D2 neurons (Figures 6I–6L and data not shown), whereas exposure to the same Activin A concentration from 24 hr to 48 hr generated D1 and D2 neurons but not neural crest cells (Figures 6M–6P and data not shown). In addition, stage 15 neural tube explants exposed to BMP4 or Activin A failed to generate neural crest cells, whereas D1 and D2 neurons were

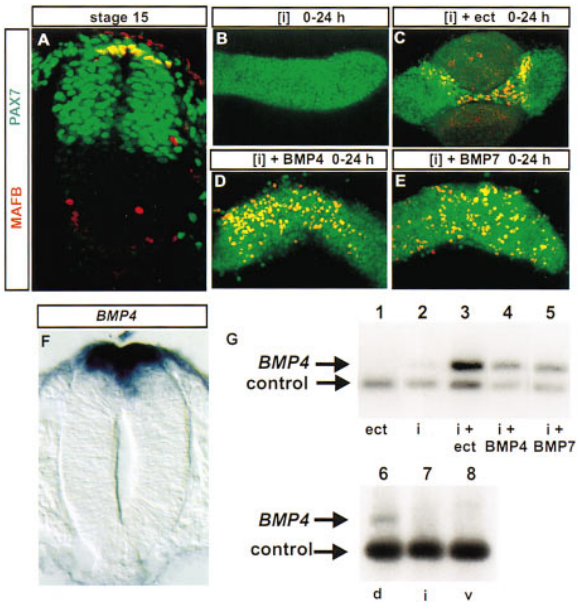

Figure 5. Induction of Roof Plate Cells by BMP-Mediated Signals from Epidermal Ectoderm

(A) Dorsal midline cells in stage 15 neural tube coexpress PAX7 (green) and MAFB (red).

Stage 10 [i] explants cultured alone for 24 hr do not give rise to MAFB/PAX7 cells (n = 7).

(C) Stage 10 [i] explants cultured with epidermal ectoderm (PAX7 negative tissue) for 24 hr generate MAFB/PAX7 cells (21 ± 6 cells/explant, n = 7) close to the ectoderm.

(D and E) Stage 10 [i] explants cultured with BMP4 (D) or BMP7 (E) for 24 hr give rise to MAFB/PAX7 cells (BMP4: 168 ± 16 cells/explant, n = 4; BMP7: 212 ± 21 cells/explant, n = 4).

(F) Stage 10 dorsal midline neural tube cells express *BMP4*.

(G) RT–PCR analysis of *BMP4* in stage 10 [i] explants. Lane 1: *BMP4* is not detected in E11 rat epidermal ectoderm (ect). *BMP4* is detected in chick epidermal ectoderm (data not shown). Lane 2: [i] explants express low levels of *BMP4* when grown for 24 hr. Lane 3: rat epidermal ectoderm tissue induces *BMP4* expression. Lane 4: BMP4 induces *BMP4* expression. Lane 5: BMP7 induces *BMP4* expression. Lanes 6–8: RT–PCR analysis of *BMP4* in explants isolated from prospective dorsal [d], intermediate [i], and ventral [v] regions of stage 10 neural plate. Dorsal but not intermediate or ventral explants express *BMP4*. Endogenous *BMP4* product is upper band and competitor mRNA is lower band. Similar results were obtained in at least three experiments.

generated (data not shown). Thus, neural plate cells exposed at early stages to TGFβ-related signals are able to generate neural crest cells but lose this capacity with time while acquiring or maintaining the capacity to generate D1 and D2 neurons. Stage 10 [i] explants that had been matured alone in vitro for 24 hr before exposure to BMP4 generated only ~20% of the number of MAFB/PAX7 cells (data not shown), suggesting that early exposure to BMPs is also required to initiate the differentiation of roof plate cells. These findings suggest that the distinct periods of generation of neural crest and roof plate cells and dorsal interneurons may be controlled, at least in part, by a temporal switch in the response of neural cells to TGFβ-related signals.

Requirement for BMPs in the Induction of Neural Crest and Roof Plate Cells by Epidermal Ectoderm

In avian embryos, BMP4 and BMP7 are the only two BMPs known to be expressed by the epidermal ectoderm (Liem et al., 1995), but their requirement in the induction of neural crest or roof plate cells has not been established. We therefore tested whether the ability of epidermal ectoderm to induce neural crest and roof plate cells can be blocked by Noggin and Follistatin. The induction of HNK1 neural crest cells (Figures 7B and 7C–7E) and MAFB/PAX7 roof plate cells (Figures 7B and 7F–7H) by epidermal ectoderm was inhibited by 80%–90% in the presence of concentrations of Noggin and Follistatin that inhibit the actions of BMP4 and BMP7 (Figure 7A and data not shown). The extent of inhibition of neural crest and roof plate cell generation was much less when conjugates were grown with either Noggin or Follistatin alone (data not shown). These results provide direct evidence that BMP signaling from the epidermal ectoderm initiates the induction of neural crest and roof plate cells.

Discussion

The origin and identity of signals that control the differentiation of neurons in the dorsal horn of the spinal cord has remained obscure. We provide evidence that BMP-mediated signals from the epidermal ectoderm induce a specialized group of dorsal midline glial cells, the roof plate, which serves as a secondary signaling center that uses multiple TGFβ-related signals to control the identity and pattern of interneuron generation in the dorsal spinal cord. We discuss the implication of these findings with reference to the contribution of epidermal ectoderm and roof plate signaling to dorsal patterning, the requirement for TGFβ-related signals in these processes, and the contrasting strategies used to pattern cell types in the dorsal and ventral halves of the spinal cord.

Requirement for BMP Signaling from Epidermal Ectoderm in the Generation of Roof Plate and Neural Crest Cells

Previous studies have shown that two BMPs expressed by the epidermal ectoderm, BMP4 and BMP7, mimic its ability to induce neural crest cells (Liem et al., 1995). The present findings extend these observation in two ways. First, the BMP antagonists Noggin and Follistatin inhibit, almost completely, the ability of the epidermal ectoderm to induce neural crest cells. This result provides strong evidence that BMPs mediate the inductive activity of the epidermal ectoderm in the chick. Since Noggin and Follistatin may antagonize the actions of several BMPs (Yamashita et al., 1995; Zimmerman et al. 1996), our results do not establish the specific contributions of BMP4 and BMP7. Nevertheless, other BMPs are not detected in the chick epidermal ectoderm (Liem et al., 1995), and thus, BMP4 and BMP7 are the strongest candidates as mediators of ectodermal signaling.

In mouse, the epidermal ectoderm expresses *BMP2*,

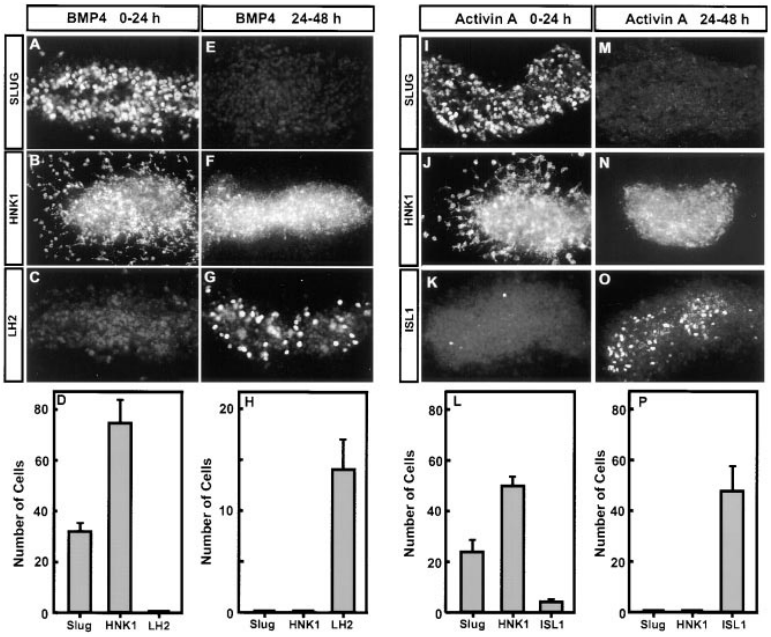

Figure 6. Temporal Switch in the Response of Neural Plate Cells to BMP4 and Activin A

(A–C) Stage 10 [i] explants cultured with BMP4 for 24 hr give rise to Slug$^+$ (A) and HNK1$^+$ cells (B) but not to LH2 neurons (C).

(D) Cell generation in response to BMP4 application from 0 to 24 hr.

(E–G) Stage 10 [i] explants cultured for 24 hr in the absence of BMP followed by exposure for 24 hr to the same concentration of BMP4 as in (A)–(C) do not generate Slug$^+$ (E) or HNK1$^+$ migratory cells (F), but generate LH2 neurons (G).

(H) Cell generation in response to BMP4 application from 24–48h. Cells/section.

(I–K) Stage 10 [i] explants cultured with Activin A for 24 hr give rise to Slug$^+$ cells (I) and HNK-1$^+$ cells (J) but few Isl1 neurons (K).

(L) Cell generation in response to Activin A exposure from 0 to 24 hr.

(M–O) Stage 10 [i] explants cultured for 24 hr in the absence of Activin A followed by exposure to Activin A for 24 hr do not generate Slug$^+$ cells (M) or HNK1$^+$ migratory cells (N), but generate Isl1 neurons (O).

(P) Cell generation in response to activin A exposure from 24 to 48 hr.

Mean ± SE; n = 4–20 explants (Isl1, HNK1) or sections (LH2, Slug).

BMP4, and *BMP7* (Lyons et al., 1995; Arkell and Beddington, 1997; Dudley and Robertson, 1997), but gene targeting studies have not yet provided evidence for the involvement of BMPs in dorsal inductive signaling in the spinal cord. Mice lacking *BMP2* or *BMP4* die during gastrulation (Winnier et al., 1995; Zhang and Bradley, 1996), and this early lethality has precluded an analysis of dorsal patterning in these mutants. Null mutations in *BMP7* lead to defects in eye development (Dudley et al., 1995; Luo et al., 1995), which may result from the loss of BMP7 in diencephalic level epidermal ectoderm. Defects in neural crest cell generation and dorsal patterning in the spinal cord have not been reported in BMP7 null mice (Dudley et al., 1995; Luo et al., 1995), which may reflect the residual activities of BMP2 and/or BMP4 (Dudley and Robertson, 1997).

Our results show also that BMP-mediated signals from the epidermal ectoderm are required for the induction of dorsal midline cells in the neural tube. At early stages of neural tube development, markers that define the roof plate (MAFB) and neural crest (Slug) are coexpressed by cells at the dorsal midline (our unpublished data). It is likely that roof plate cells and neural crest cells are generated from a common progenitor cell that occupies the dorsal midline of the neural tube. In support of this, lineage tracing studies in chick (Bronner-Fraser and Fraser, 1988) and mice (Echelard et al., 1994) have provided evidence that cells at the dorsal midline of the neural tube can give rise both to neural crest and roof plate cells.

It remains unclear how the distinct identities of roof plate and neural crest cells are established. Temporal changes in gene expression by dorsal midline cells might contribute to this distinction. The transcription factor Slug has been suggested to promote the emigration of neural crest cells from the dorsal neural tube (Nieto et al., 1994) and is expressed transiently (Nieto et al., 1994; Liem et al., 1995). The extinction of Slug expression from dorsal midline cells could contribute to the loss of potential of cells to generate neural crest with the consequence that MAFB cells remain at the dorsal midline of the neural tube and acquire roof plate identity.

Requirement for TGFβ-Related Signals from the Roof Plate in Dorsal Interneuron Generation

The differentiation of roof plate cells is accompanied by the expression of several members of the *BMP* family and of *Activin B*. Three lines of evidence indicate that the roof plate serves as a secondary source of TGFβ-related signals involved in dorsal interneuron generation. First, signals from the roof plate are sufficient to induce both D1 and D2 neurons. Second, TGFβ-related signals mimic the ability of the roof plate to induce these two classes of neurons. Third, the blockade of TGFβ-like signals from the roof plate markedly inhibits the differentiation of D1 and, to a lesser degree, D2 neurons. Our results do not exclude that a component of the inductive activity of the roof plate could be mediated by factors other than TGFβ-related proteins.

Nevertheless, taken together with the demonstration of a requirement for BMPs in the induction of neural crest and roof plate cells, our results indicate that the generation of the diverse cell types found in the dorsal half of the neural tube and spinal cord depends in large part on TGFβ-related signals. These inductive interactions appear to involve a cascade of TGFβ-mediated signals initiated by the epidermal ectoderm and propagated by roof plate cells. The relative contributions of

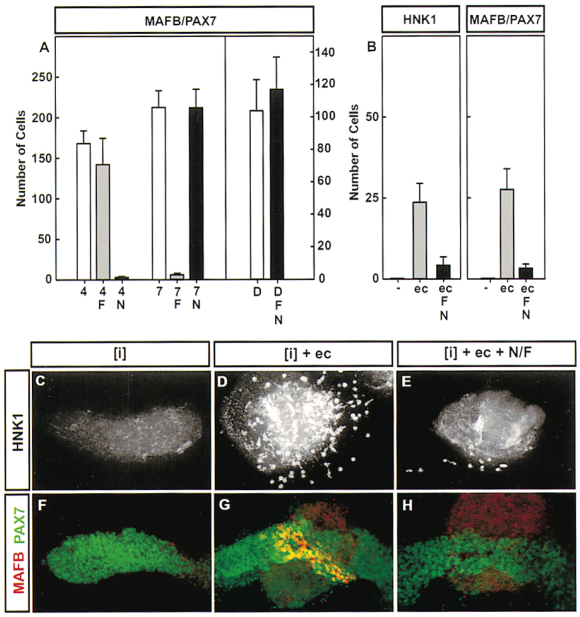

Figure 7. Requirement for BMP Signaling in the Induction of Neural Crest and Roof Plate Cells by Epidermal Ectoderm

(A) Selectivity of Noggin (20 nM) and Follistatin (85 nM) actions in the blockade of MAFB/PAX7 induction by BMP4, BMP7, and DSL1. Mean ± SE; n = 4–20 explants. Similar results were obtained for HNK1 expression (data not shown).

(B) Blockade of neural crest (migratory HNK1) and roof plate (MAFB/PAX7) induction by epidermal ectoderm with Noggin (20 nM) and Follistatin (85 nM). Mean ± SE; n = 4–20 explants.

(C–E) Noggin and Follistatin inhibit the induction of HNK1$^+$ neural crest cells by epidermal ectoderm.

(F–H) Noggin and Follistatin inhibit the induction of MAFB/PAX7$^+$ cells by epidermal ectoderm. Abbreviations are as in Figure 4.

epidermal ectoderm and roof plate signaling to the generation of specific dorsal cell types in vivo remain unclear. However, the timing of gene expression and cell differentiation suggests that ectodermal signals direct neural crest and roof plate cell fates and that roof plate signals direct dorsal interneuron fates.

Temporal and Spatial Control of Cell Differentiation in the Dorsal Spinal Cord

How is the temporal and spatial control of cell differentiation by multiple TGFβ-related signals achieved?

One determinant of the distinct periods of neural crest and dorsal interneuron generation appears to be a temporal switch in the response of neural progenitor cells to TGFβ-related signals. At early stages of development, neural cells respond to BMPs with the generation of neural crest cells but not of dorsal interneurons, whereas at later stages the same BMP signal elicits the generation of dorsal interneurons but not of neural crest cells. This temporal switch might therefore contribute to the early onset and transience of neural crest cell generation (Newgreen and Erickson, 1986) and the later onset and prolonged duration of dorsal interneuron generation (Oppenheim et al., 1988) observed in vivo.

The identity and position at which D1 and D2 neurons are generated appear to depend in part on the time at which neural cells are exposed to BMPs. Cells exposed to BMPs at an early stage of neural tube development are able subsequently to generate D1 neurons but are inhibited from generating D2 neurons. Since BMP signaling emanates from cells in and around the roof plate, this finding suggests that the more dorsally located a progenitor cell, the earlier and more prolonged will be its exposure to BMPs and the greater the probability that it will generate a D1 neuron.

How then are D2 neurons induced? Our results show that dorsal progenitor cells that escape early exposure to BMPs can respond later to BMPs with the generation of D2 neurons. Indeed, under these conditions, cells exposed to low BMP concentrations generate D2 but not D1 neurons. The nested patterns of *BMP* gene expression in vivo suggest the existence of a dorsal-to-ventral gradient of BMP activity in the dorsal spinal cord. Such a gradient would be expected to lead to the generation of D1 neurons dorsally and D2 neurons more ventrally. By analogy with BMP signaling in other systems, a gradient of BMP activity could be established by a relay mechanism that uses short-range BMP signaling to induce other *BMP* genes (Reilly and Melton, 1996) and/or by a longer-range diffusion of BMP proteins (Gurdon et al., 1994; Jones et al., 1996; Lecuit et al., 1996; Nellen et al., 1996).

An additional factor in the diversification of D1 and D2 neurons appears to be a qualitative difference in the signaling activities of TGFβ-related proteins expressed in and around the roof plate. Although the Activin-induced generation of D2 neurons appears to be suppressed at dorsal positions by the presence of BMPs, there is a broad domain of expression of *Activin B* in the dorsal spinal cord (Figure 2, see also Feijen et al., 1994). In addition, the range of diffusion of Activin appears to be greater than that of BMPs (Jones et al., 1996). Thus, a domain of Activin B activity broader than that of BMPs may also contribute to the generation of D2 neurons at a position ventral to that of D1 neurons. Spatial and temporal controls on the expression of receptors of the TGFβ family and of SMADs or other mediators of TGFβ signaling (Massague, 1996) are likely to underlie the distinct cellular responses of neural progenitor cells to different members of the TGFβ superfamily.

Comparison of Dorsal and Ventral Patterning in the Neural Tube

The present results indicate that there are differences in the strategies by which cell fate and pattern are regulated in the dorsal and ventral halves of the neural tube. First, ventral patterning in higher vertebrates is regulated by the activities of a single Hedgehog protein, Shh (Tanabe and Jessell, 1996). In contrast, dorsal patterning appears to involve multiple members of the TGFβ family. Second, Shh appears to control ventral cell fate and pattern primarily through its ability to induce distinct cell types at different concentration thresholds (Roelink et al., 1995; Ericson et al., 1996, 1997). In contrast, qualitative differences in the activities of members of different subclasses of the TGFβ family appear to contribute to the control of cell type and pattern in the dorsal neural tube. Third, a temporal change in the response of neural cells to BMPs appears to contribute to the diversification

of dorsal cell types, whereas the competence for the generation of distinct ventral cell types in response to Shh is lost synchronously (Ericson et al., 1996, 1997).

There is, however, one common feature in the deployment of signals that control dorsal and ventral cell pattern. Inductive signals are expressed initially by nonneural tissues and are then transferred, through a process of homeogenetic induction, to specialized glial cells at the dorsal and ventral midlines of the neural tube. This feature presumably ensures that a local source of inductive signals is positioned appropriately for the control of neural cell fate and pattern at later times, when the original source of these factors is no longer available. Finally, emerging evidence has implicated BMPs in dorsal cell differentiation at more rostral levels of the neuraxis (Arkell and Beddington, 1997; Furuta et al., 1997; Muhr et al., 1997). The mechanisms by which TGFβ-related proteins control cell identity and pattern in the spinal cord may therefore operate more widely.

Experimental Procedures

Isolation of cDNA Clones

A chick brain λgt11 cDNA library (Clontech) was screened with rat *LH2A* cDNA (Xu et al., 1993) to obtain a *LH2B* cDNA (GenBank accession number L35566). A chick *LH2A* cDNA provided by J.C. Izpisua-Belmonte was used to show that *LH2A* is expressed in the spinal cord in a pattern similar to that of *LH2B* (our unpublished data). cDNAs encoding chick *BMPs* were isolated as described (Basler et al., 1993; Liem et al., 1995).

Antibody Generation

A chick *LH2B* cDNA encoding the homeodomain and C terminus was cloned into pGEX (Pharmacia). A 45 kDa fusion protein was used to generate a rabbit anti-LH2 Ab that recognizes LH2B and LH2A (data not shown). A chick *Slug* cDNA (Nieto et al., 1994) was used to generate MAbs 1E6 and 1F4.

In Situ Hybridization Histochemistry

In situ hybridization histochemistry was performed as described by Harland (1991) and Schaeren-Wiemers and Gerfin-Moser (1993).

RT–PCR

RT–PCR was performed as described (Tanabe et al., 1995). Oligonucleotides to amplify BMP4 were 5′ CAGGAGATCAGCCTGCAGTAC CCG 3′ and 5′ CCTGAGCTGCGCCCAGTCCCCGCC 3′.

Induction Assays

Neural plate, neural tube, and epidermal ectoderm tissue from Hamburger and Hamilton (1951) stage 10–15 chick embryos and ectodermal tissue from E11 rat embryos were isolated and cultured as described (Yamada et al., 1993; Liem et al., 1995).

Mouse BMP4, human BMP7, chick DSL1, chick truncated DSL1, and human Activin A were obtained by transfection of COS cells (Basler et al., 1993). BMP4, BMP5, and BMP7 from CHO cells (provided by Genetics Institute) were used in some assays. Xenopus Noggin was provided by R. Harland. Follistatin was obtained from the National Hormone and Pituitary Program.

Dorsal Notochord Grafts and Notochord Removals

Notochord manipulations were performed according to Yamada et al. (1991). Analyses were performed after 72 hr.

Immunocytochemistry

Immunocytochemistry was performed as described (Yamada et al., 1991, 1993). LH2A/B was detected with rabbit Ab L1, Isl1 with MAb 4D5, or rabbit Ab K5 (Tsuchida et al., 1994), Isl2 with MAb 4H9 (Tanabe et al., 1995), MAFB with a rabbit Ab (Puoponnot et al., 1995), Pax7 with a MAb (Ericson et al., 1996), and chick TAG1/Axonin-1 with MAb 4-5. MAb QCPN labels quail tissue (Liem et al., 1995). MAb HNK1 recognizes neural crest cells (Tucker et al., 1984). MAb FP1 recognizes floor plate cells (Yamada et al., 1991).

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